ANTARCTIC FISH CHROMOSOME BANDING: SIGNIFICANCE FOR EVOLUTIONARY STUDIES *

by

Catherine OZOUF-COSTAZ (1), Eva PISANO (2),
Christelle THAERON (1) and Jean-Claude HUREAU (1)

ABSTRACT. - An overview of the results obtained with banding techniques which could be applied to Antarctic fish chromosomes till now is presented. This includes nucleolar organizers (NORs) staining by silver nitrate and chromomycin A_3, C-banding and fluorescence *in situ* hybridization of molecular probes. The aim is to show their possible applications in comparative cytogenetics and evolutionary studies. In the light of some examples taken in our results, we try to demonstrate why the chromosome characters should be more informative when used within the lowest taxonomic levels, than for establishing phylogenies at family level.

RÉSUMÉ. - Marquage des chromosomes de poissons antarctiques: application pour les études de phylogénie.

Un récapitulatif des résultats obtenus avec les techniques de marquage (banding) qu'il a été possible d'appliquer jusqu'à présent aux chromosomes des poissons antarctiques est exposé. Il s'agit en particulier de la coloration des régions porteuses des organisateurs nucléolaires (NORs) par le nitrate d'argent et la chromomycine A_3, du banding C et de l'hybridation en fluorescence de sondes moléculaires. La finalité est de montrer leurs applications possibles pour la cytotaxinomie et la phylogénie. En nous appuyant sur certains exemples choisis parmi nos résultats, nous tentons de démontrer pourquoi les caractères chromosomiques sont plus susceptibles d'être instructifs et discriminants aux rangs taxinomiques inférieurs que pour l'établissement de phylogénies de familles.

Key-words. - Notothenioidei, PSE, Antarctic Ocean, Chromosome banding, Fluorescence *in situ* hybridization (FISH), Taxonomy, Phylogeny.

Over the past decade, karyological investigations have been carried out on notothenioid species by Russian, French, Brazilian and Italian scientists in various sectors of the Southern Ocean. Standard karyotypes (chromosome numbers and formulae) are known for about 60 species, mostly Channichthyidae and Nototheniidae (see the reviews by Ozouf-Costaz *et al.*, 1991; Prirodina, 1994). All authors agree that a karyotype of 48 acrocentric chromosomes is primitive for notothenioid fish as for other Perciformes (Ohno, 1974; Sola *et al.*, 1981). This diploid number is statistically the most frequent and occurs in all families except Artedidraconidae. The position of centromeres allowed to establish chromosome formula and in some cases, to identify individual chromosome

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(1) Muséum national d'Histoire naturelle, Laboratoire d'Ichtyologie générale et appliquée, 43 rue Cuvier, 75231 Paris Cedex 05, FRANCE.

(2) Università di Genova, Istituto di Anatomia Comparata, Viale Benedetto XV, 5, 16132 Genova, ITALIA.

pairs in a given karyotype. The most frequent rearrangements were described on the basis of morphology: one of the most widespread differentiation process is a progressive reduction of chromosome number by centric fusions of acrocentrics thus providing metacentrics, but there are obviously also some other types of chromosome changes which do not affect the number, but only the formula, such as translocations, para- or pericentric inversions and other processes such as addition of heterochromatin (Pisano *et al.*, in press). Heteromorphism linked to multiple sex chromosome systems has also been described in some species of Channichthyidae, Nototheniidae and one Bathydraconidae (Ozouf-Costaz *et al.*, 1991; Morescalchi *et al.*, 1992a, 1992b). In some notothenioid families (Channichthyidae, Artedidraconidae), gross karyotype change appears to have been minimal. Similar mechanisms of karyotype differentiation occurred separately in the families and genera, due to preferential sites of rearrangement, thus leading to identical chromosome numbers and formulae. Moreover, comparisons between species were not very informative because chromosomal homologies could not be ascertained. It is therefore impossible to find, on the sole base of chromosome numbers and morphologies, any apomorphic feature unique to Notothenioidei or unique to a family. However, the more recent development of chromosome banding on Antarctic fish chromosomes seems more promiseful, since it allows their longitudinal differentiation and thus their unequivocal identification. Such methods require consistently good chromosome spreads and this limiting factor can explain their later development in Antarctic fishes. Banding patterns can be used to study chromosomal changes that have occurred between related species, so we expect that the identification of chromosomal rearrangements within subfamilies or genera might be progressively possible. We also expect that minor chromosome changes may allow population characterization. On the contrary, there is no evidence that such chromosome features could discriminate at higher taxonomic levels.

Here we present an overview of the banding techniques which could be applied to Antarctic fish chromosomes till now, with some examples selected in order to show their possible applications in comparative cytogenetics and evolutionary studies. From these results, we try to withdraw what more should be done for increasing our knowledge of karyoevolutionary processes in this group. For examples taken from unpublished data, details on the material and methods will be published elsewhere.

Nucleolar organizers (NORs)

NORs contain in numerous repeats ribosomal genes clusters, usually localized in a small number of chromosomal positions. At the molecular level, these genes are highly conserved among eukaryotes, for example the conserved core of the 28s rDNA (Raue, 1988; Qu *et al.*, 1988). This allows the design of conserved primers for universal PCR amplificates and therefore suitable probes for *in situ* hybridization (FISH). As stressed by Amemiya and Gold (1988), since « NORs are under strong functional and organizational constraint [...] inter-specific chromosomal rearrangements involving NORs may be systematically or phylogenetically informative ».

Ag-NORs silver staining

In the case of Antarctic fish, staining of the NORs has been mostly done with silver. The method stains active NORs (major NORs) that are synthesizing ribosomal RNA rather than every region containing rDNA sequences; thus not all the NORs are necessarily stained. Some examples of Ag-*NORs* staining in Antarctic fish can be found in Morescalchi *et al.* (1992a, 1992b); Pisano *et al.* (1995) and Ozouf-Costaz *et al.* (1996).

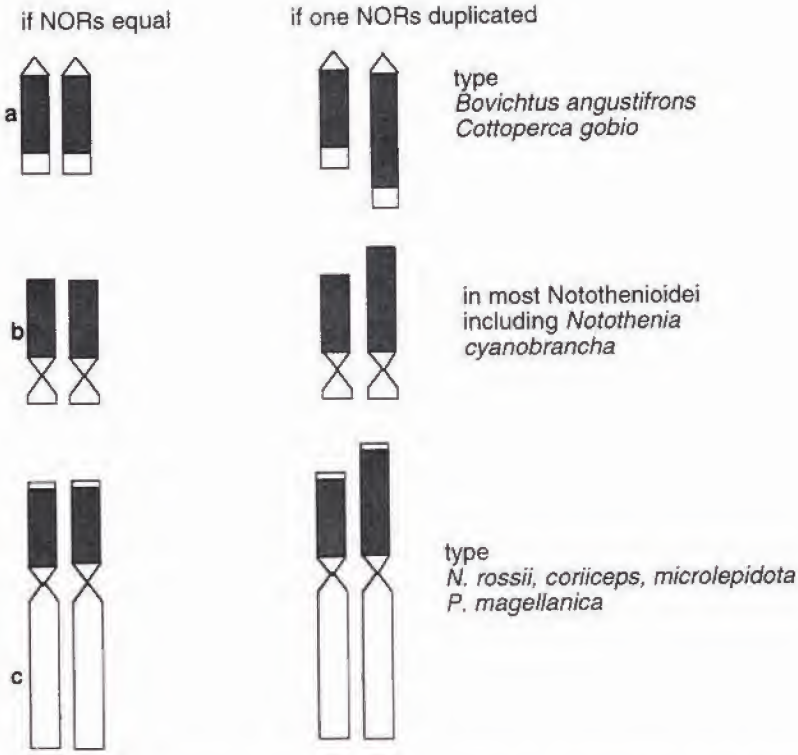

Fig. 1. - Major NORs locations in the karyotyped Notothenioidei: a) pericentromeric in an acrocentric chromosome pair; b) almost entirely constituting the long arm of a small submetacentric chromosome pair; c) interstitial in the short arm of a large submetacentric pair. N.B. NORs can be duplicated in one of the two chromosomes of the pair.

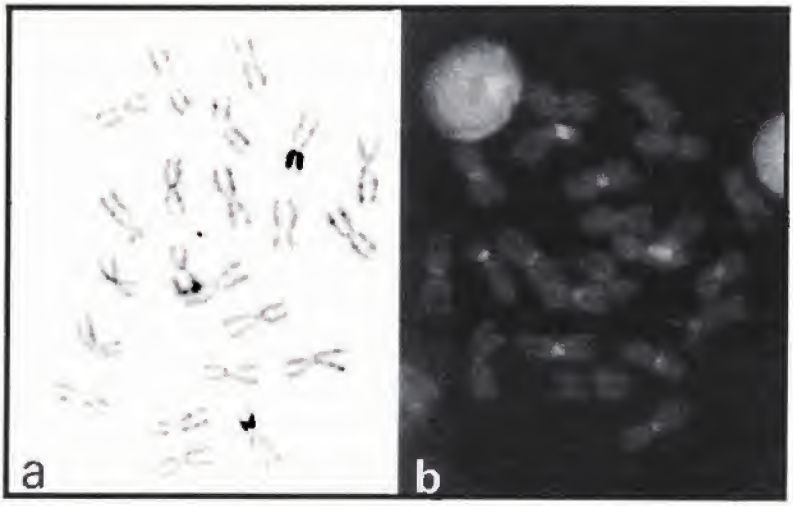

Fig. 2. - NORs location as revealed by silver staining (a) and CMA_3 (b) in the chromosomes of *Notothenia coriiceps*. Scale bar = 10 µm.

NORs silver-staining is also the banding technique which has been the most widely applied to Antarctic fish chromosomes. In some groups, there are enough data for using NORs location as character for phylogenetic purposes. For example, in two bovichtid species *Cottoperca gobio* (2n = 48 acrocentrics) and *Bovichtus angustifrons* (2n = 48 acrocentrics, unpublished), NORs are located in pericentromeric position in a medium size acrocentric pair (Fig. 1a) which may represent the NORs ancestral position, since it is now assumed that the ancestral perciform karyotype is 2n = 48 acrocentrics. In the majority of other notothenioid species investigated (a few Channichthyidae, and some Nototheniidae), active NORs are located on the short arms of a medium size pair of submetacentric chromosomes (Fig. 1b). However, we found another NORs location when examining the chromosomes of the species of the two genera *Notothenia* and *Paranotothenia*. In the karyotype of *Notothenia coriiceps* from the Ross Sea (Morescalchi *et al.*, 1992b) and from Adelie Land (present paper), NORs are inserted in the centromeric region of a large pair of submetacentric chromosomes (Fig. 1c; Fig. 2a). We also examined the chromosomes of two closely related species *N. rossii* (2n = 24) and *Paranotothenia magellanica* (2n = 26). Although the two species are considered to belong to two different genera on the basis of osteological characters (Balushkin, 1976, 1984), their morphological similarities are pointed out by DeWitt *et al.* (1990). Karyotypes of the two species are also very similar and have been studied by Doussau de Bazignan and Ozouf-Costaz (1985). Standard karyotype of *N. rossii* could be completed by G- and C-banding. There is a large heterochromatic block in the first pair, the length of this band being, like in *N. coriiceps* from Adelie Land, unequal in size within the pair, which undoubtedly corresponds to the NORs. In *P. magellanica*, C-banding could not be performed, but NORs, which are often apparent as secondary constriction of the chromatids at metaphase, are also located at the same position in the first submetacentric pair. Although the NORs position has not been studied in all species belonging to the genus *Notothenia*, it is to be pointed out that *Notothenia microlepidota* (2n = 26) has exactly the same chromosome formula as *P. magellanica*, the first pair being a large submetacentric, heteromorphic (Prirodina, 1984). On the contrary *Notothenia cyanobrancha* (Doussau de Bazignan and Ozouf-Costaz, 1985) has a very different karyotype (2n = 48) and its NORs located in the b) position.

In the data matrix summarizing the NORs patterns for the *Notothenia / Paranotothenia* group (Table I) the NORs pattern a) being considered as plesiomorphic, the two Bovichtidae *Cottoperca gobio* and *Bovichtus angustifrons* are used as outgroup. The identified patterns b) and c) which are supposed to result from particular chromosome

Table I. - Data matrix based on the presence/absence of b) and c) characters coded as 1/0; d) character is defined as presence/absence of acrocentric chromosome coded as 0/1.

Taxa	Characters		
	b	c	d
Cottoperca gobio	0	0	0
Bovichtus angustifrons	0	0	0
Notothenia cyanobrancha + most karyotyped Notothenioidei	1	0	0
Notothenia coriiceps	1	1	1
Notothenia rossii	1	1	1
Notothenia microlepidota	1	1	0
Paranotothenia magellanica	1	1	0

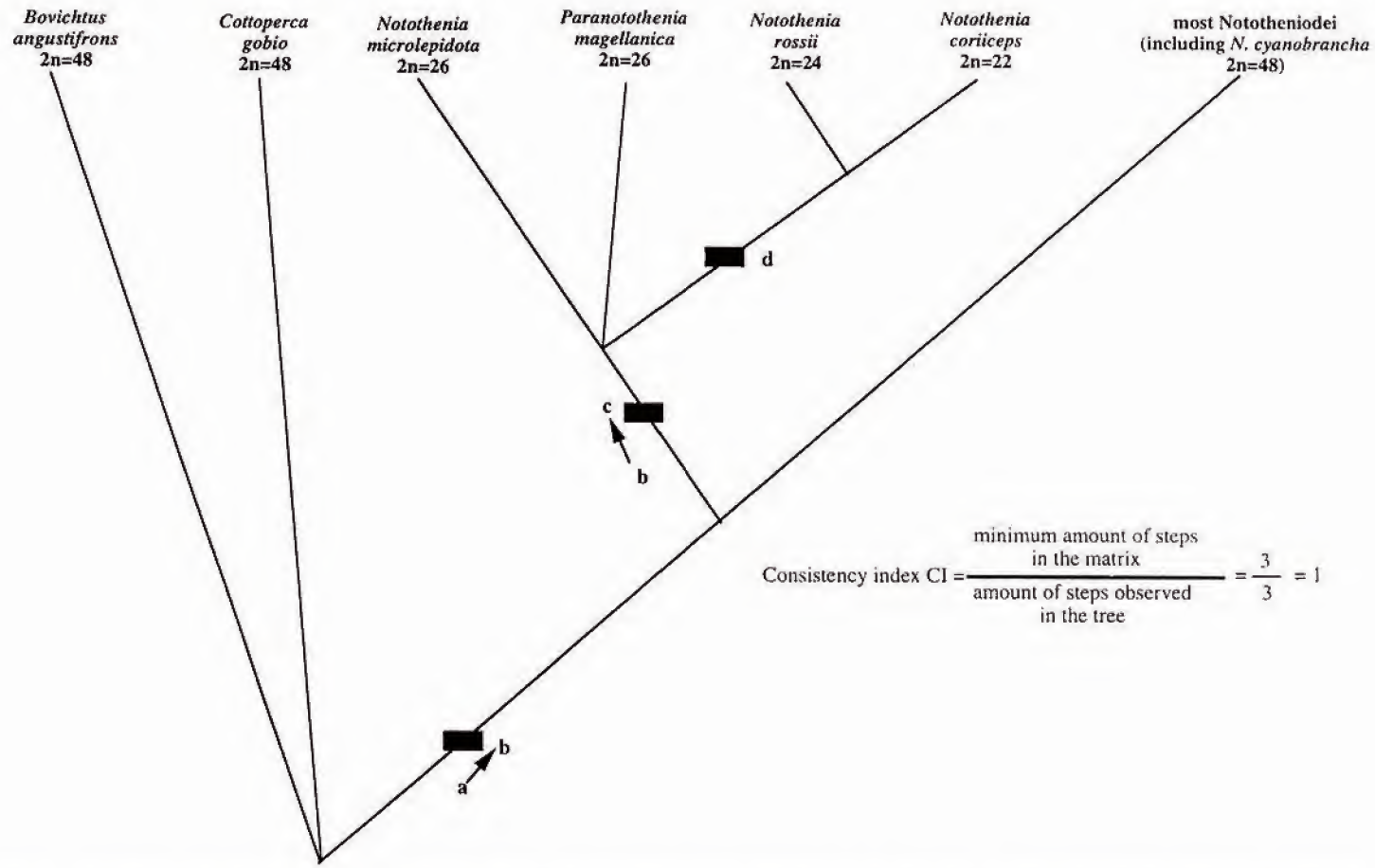

Fig. 3. - Phylogenetic tree of relationships within the *Notothenia / Paranotothenia* group established on the base of b), c), and d) karyological characters as defined in the matrix of table I. *Bovichtus / Cottoperca* have been chosen as outgroup. Chromosome diploid numbers have been mapped on the tree. Black squares indicate the rearrangements inferred from the tree topology, that are likely to have lead to the changes of states a>b>c.

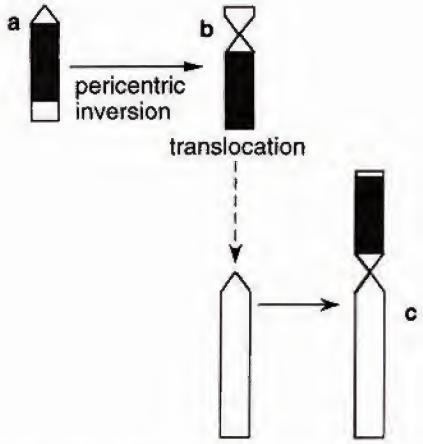

Fig. 4. - Hypotheses concerning major NORs positions and their evolution within the suborder Notothenioidei. Inference for the group *Notothenia / Paranotothenia*.

rearrangements have been coded as character states by assigning presence/absence (1/0) without polarization. In order to avoid to weight twice a particular event (or character), because affecting two chromosomes per karyotype, we systematically considered each pattern *as a character state in the haploid set*. We have identified a third character d) as presence / absence of acrocentric chromosomes in the karyotype, coded 0/1.

From this matrix can be deduced only one tree since the consistency index CI = 1 (Fig. 3). In this tree, the taxa *coriiceps* (2n = 22), *rossii* (2n = 24) and *magellanica / microlepidota* (2n = 26) are clustered on the base of their NORs in c) position, while *cyanobrancha* (2n = 48) is clustered with other karyotyped Nototheniidae. On the basis of d) character, *rossii* and *coriiceps* appear to be sister species and more closely related to each other than to *magellanica* or *microlepidota*. From the topology of this tree it is possible to withdraw hypothesis concerning chromosome rearrangements leading to changes in NORs position (Fig. 4): the b) position could result from the pericentric inversion of the previously telocentric NORs bearing pair a). The intercalated location of NORs c) should correspond to the translocation of the previously submetacentric NORs bearing pair b) on a large acrocentric pair.

So, on the base of this phylogenetic tree, *coriiceps/rossii*, *magellanica* and *microlepidota* could be subgenera of the genus *Notothenia*, and the species *cyanobrancha* could be re-assigned to another genus, *Indonotothenia*, according to the former suggestion of Balushkin (1976). DeWitt *et al.* (1990) also underline that *N. cyanobrancha* seems less closely related to the other species of *Notothenia* than they are to one another.

With regard to the absolute number of Ag-NORs per species, Hsu *et al.* (1975) and Schmid (1978) have suggested that a single homologous pair of Ag-NORs is a primitive state for Vertebrates. If so, this means that cases of multiple NORs bearing chromosomes as encountered in *Pseudaphritis urvillii* (Pisano *et al.*, 1995), *Pagetopsis macropterus* (Morescalchi *et al.*, 1992b), and several nototheniid species (unpubl. data) can be considered as autapomorphies. So both Ag-NORs location and numbers are likely to be used for systematic and phylogenetic inference. We also expect that NORs numbers and locations could be used, in some species, as population markers because, for instance, the NORs number in *Trematomus hansoni* (unpubl. data, and see Fig. 5) from Adelie Land is cons-

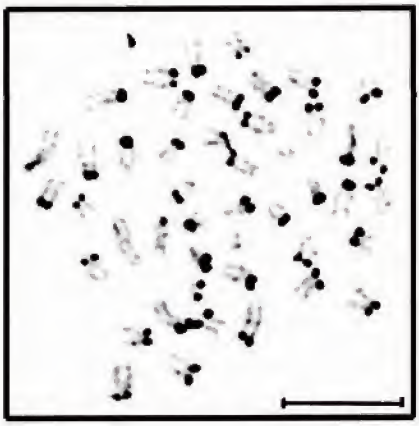

Fig. 5. - Multiple NORs location in the chromosomes of *Trematomus hansoni* from Adelie Land. Scale bar = 10 µm.

tant and much higher than for the same species from the Ross Sea (Morescalchi *et al.*, 1992a).

Chromomycin A_3 (CMA_3) staining

In lower vertebrates, chromomycin A_3, which is a GC-specific fluochrome, is believed to allow visualization of chromosomal NORs sites regardless activity. However, recent cytogenetic studies in fishes indicate that not all CMA_3 positive sites represent rDNA cistrons (Martinez *et al.*, 1991). In Gymnotoidei, Foresti de Almeida Toledo *et al.* (1996) underline that CMA_3 stains GC-rich regions not related to NORs. Apparently, this problem will require much deeper investigations. Meanwhile, CMA_3 staining has been performed on some species of Notothenioidei with various results: in *Champsocephalus gunnari* (Ozouf-Costaz *et al.*, 1996) and *Eleginops maclovinus* (Ozouf-Costaz *et al.*, unpubl. data), CMA_3 strictly corresponds to AgNORs location. In *Chionodraco hamatus* from the Ross Sea which has one pair of Ag-NORs, two pairs of chromosomes have positive CMA_3 signals (Morescalchi *et al.*, 1992b). In the same species from Adelie Land, all pairs have CMA_3 positive signals except sex-linked chromosomes. In *N. coriiceps* (Fig. 2b) the number of CMA_3 positive bands is much higher, including not only Ag-NORs location, but centromeric, juxtacentromeric or telomeric position in some pairs. So this technique is likely to give much more information than the NORs location itself. The main problem in using chromosomal NORs and CMA_3 sites for phylogenetic purposes comes from the difficulty to ascertain chromosome homologies among taxa.

C-banding

C-banding, which stains constitutive heterochromatin, works in fish and can be an important tool for chromosome identification. Examples of such bandings in Antarctic fish are shown in figure 6. The C-bands can vary significantly in size between species, and sometimes within a pair of homologous chromosomes. Heterochromatin can also be located in intercalate or terminal position instead of centromeric, which can be interpreted as footprints of a chromosome rearrangement.

In fish, Ag-NORs are C-band positive but CMA_3 bands do not systematically correspond to C-bands, so the combination between the two patterns (CMA_3 and C-banding) might be useful for karyotype comparisons between closely related species, since they

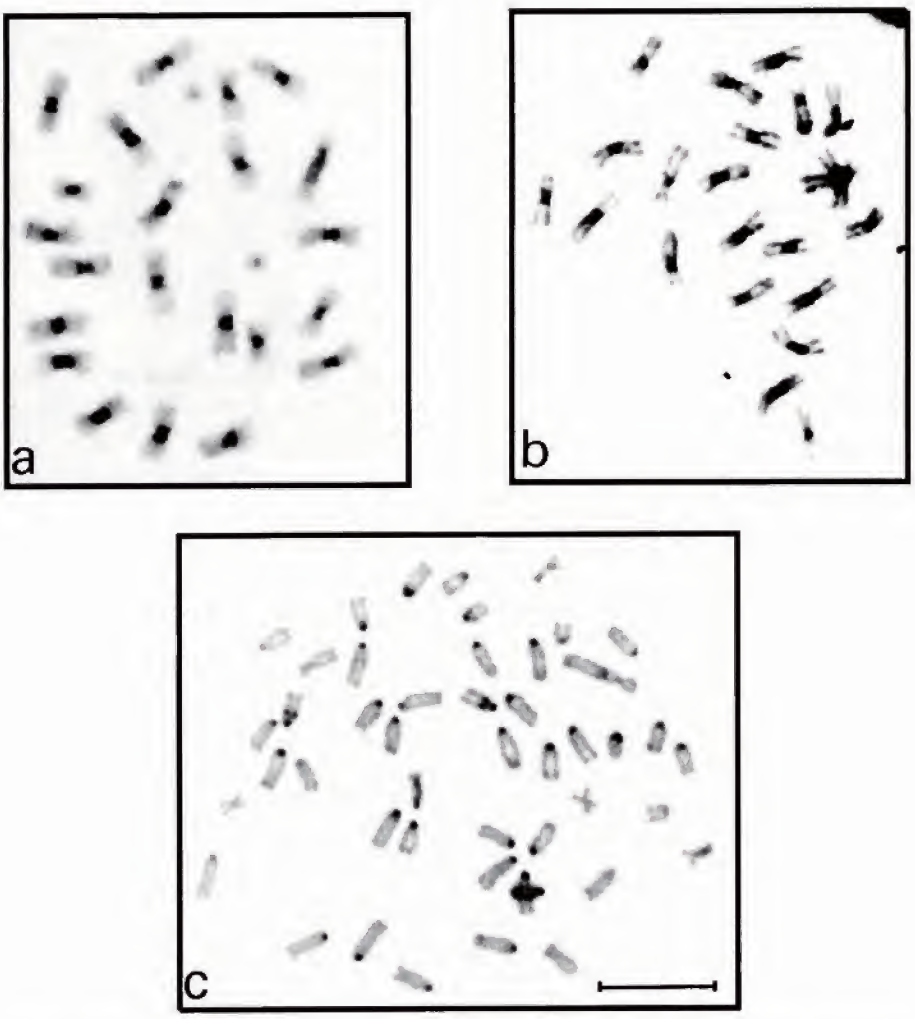

Fig. 6. - Some examples of C-banding in some species of Notothenioidei. a) *Notothenia rossii* from Kerguelen; b) *N. coriiceps* from Adelie Land; c) male of *Chionodraco hamatus* from Adelie Land. Scale bar = 10 µm.

reveal different classes of repeated sequences. For instance, the patterns of positive C-banding in some Channichthyidae suggest a possible way of karyotype differentiation by addition of heterochromatin (Pisano *et al.*, in press). In this family, the karyotype is apparently conserved regarding chromosome number and formulae only (Prirodina, in press), but our first results with C-banding and Ag-NORs staining clearly show that numerous rearrangements occurred, without affecting chromosome morphology.

Fluorescence *in situ* hybridization (FISH)

FISH, when combined with banding, makes it possible to localize DNA fragments of various size in chromosomes. This method is more sensitive than CMA_3 and silver staining in detecting sites containing only a few copies of rDNA (Pendas *et al.*, 1993; Reed and Phillips, 1995; Foresti de Almeida-Toledo *et al.*, 1996). The feasibility of this method on Antarctic fish chromosome has been demonstrated on *Champsocephalus gunnari* (Ozouf-Costaz *et al.*, in press) using a ribosomal probe. In *C. gunnari*, silver staining, CMA_3 and FISH revealed the same location for ribosomal genes.

Beside the localization of multicopy genes such as ribosomal DNA, repetitive sequences are also easier to visualize in fish chromosomes than single copy sequences. This might be useful to study chromosome rearrangements or deletions. The most widely used in fish is the telomeric sequence $(TTAGGG)_n$ which is conserved in almost all vertebrates: the interstitial sites of this sequence are interpreted as being the « footprints » of previous chromosomal fusions (see the review by Phillips and Reed, 1996). Attempts for FISH of telomeric probes on the chromosomes of Antarctic fish are currently done by our team.

Centromeric and subtelomeric sequences are often species-specific, and some could be isolated from fishes and used as probes, such as a 1000 bp centromeric/telomeric specific monomer isolated in the icefish *C. hamatus* (Capriglione *et al.*, 1994).

Another promiseful perspective in molecular cytogenetics is the possibility of preparing probes from entire chromosomes or chromosomes arms obtained by microdissection and PCR amplified. Reed *et al.* (1995) successfully produced a probe derived from the short arm of the lake trout Y chromosome. Such a probe can then been hybridized on the chromosomes of related species in order to produce a « chromosome painting ». Comparisons between the paintings could help to ascertain inter-specific homologies. Such techniques are now widely developed in human cytogenetics.

CONCLUSION

Consequently, to get homologous characters, banding techniques combined with molecular cytogenetics should be useful to highlight Antarctic fish relationships. We also believe that the chromosomal characters should be more informative when used within lowest taxonomic levels, in contrast with molecular data (DNA sequencing) that have been, up to now, more widely used to establish phylogenies at family level.

For instance at infraspecific level, *Trematomus eulepidotus* has $2n = 24$ metacentric chromosomes in the Weddell Sea (Ozouf-Costaz *et al.*, 1991) and the Ross Sea (Morescalchi *et al.*, 1992b) but the few specimens studied in Prydz Bay (Ozouf-Costaz and Doussau de Bazignan, 1987) have $2n = 22$ metacentrics and 2 medium size acrocentrics. C-banding or *in situ* hybridization of a telomeric probe may allow to ascertain chromosomal homologies and to understand what chromosome change occurred in Prydz Bay population. NORs numbers and locations could also be used either as population markers in some species. Chromosomal rearrangements, when found fixed in population, could be a first step towards complete reproductive isolation

At peri-specific levels, and specially in genera or families in which there is low variation in chromosome numbers and formulae, it is very probable that NORs position or number can also be used for characterizing some taxonomic units such as for the genus *Notothenia.* Besides the NORs, other banding techniques and FISH with various probes

should bring much more characters to be used to highlight fish relationships at interspecific levels. Indeed, the fact that closely related species or populations differ in karyotypes may support the idea that some chromosomal changes may be important in speciation (see King, 1994). However, in Antarctic fish it still remains difficult to understand if it is chromosomal difference which promoted speciation or whether it appeared incidentally before or after species formation.

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